Physiological Responses of Amaranthus Cruentus L. to Drought Stress Under Sufficient- and Deficient-Nitrogen Conditions

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Physiological responses of *Amaranthus cruentus* L. to drought stress under sufficient- and deficient-nitrogen conditions

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ABSTRACT

Water and nitrogen availability are environmental factors that can impair plant growth, and when they are combined their effects can be intensified or reduced. The objective of this study was to analyse the influence of nitrogen availability on the responses of *Amaranthus cruentus*'s metabolisms to water stress. The plants were cultivated in plastic pots filled with vermiculite and kept under greenhouse conditions and were watered with 70% of full strength nitrogen-free Long Ashton solution, containing 1.97 or 9.88 kg N ha^{-1} as ammonium nitrate, three times a week. Photosynthetic parameter were evaluated *in planta* and leaves were harvested for chemical analysis of proline and phenolic contents. Higher nitrogen supply increased the shoot dry matter, photosynthetic pigments, photosynthesis, stomatal conductance, transpiration, total leaf nitrogen, proline, nitrate and ammonium but reduced the concentration of flavonoids and total phenols. Water stress for 6 days did not affect dry matter, photosynthetic pigments, leaf nitrogen, ammonium or specialized metabolites but increased the proline and affected negatively the other variables. The observed interactions between nitrogen and water supply resulted in no alleviation of the negative effects of drought on amaranth. Although the increase in nitrogen supply had benefits on plant performance, it intensified the negative effect of water stress. The study shows the importance of choosing the correct level of nitrogen fertilization in order to obtain satisfactory results in terms of plant growth under drought conditions.

Introduction

Nitrogen is an essential nutrient for plant development and its deficiency reduces growth and photosynthesis because the large amount of total leaf nitrogen is located in the chloroplasts[1]. Mu and Chen[2] demonstrated that C_4 plants invest more nitrogen into light harvesting protein suggesting that these plants have a higher light energy convention and electron transport rate. A higher leaf nitrogen allocation is expected in most plants well-nourished with nitrogen and in a positive correlation with photosynthetic capacity in both C_3 and C_4 plants[3,4]. Due to increased global warming, climate change and the risk of extreme events such as prolonged droughts are expected to increase in the coming years, with the rural areas estimated to have major impacts on water availability and supply[5], resulting in losses in agriculture. It is well known that water deficit is considered one of the major abiotic stress affecting plant productivity due to its negative effects on plant growth[6] and photosynthesis[7]. Under mild water stress, the leaves close their stomata in order to save water and improve water-use efficiency. However, with the closure of the stomata, there is also a reduction in CO_2 supply to the leaves, and, as a consequence, a reduction in the capacity for CO_2 assimilation.

Plants under water restriction are able to synthesise and accumulate several osmolytes in the cells in an attempt to reduce the osmotic potential. In a recent review about osmolyte synthesis and accumulation it was shown that they belong to several classes of compounds such as sugars, polyamines, and amino acids as proline with all these osmolytes involved in reactive oxygen species (ROS) scavenging, osmotic adjustment, improving assimilation of CO_2 and protection of membrane [8, and references therein]. Therefore, the osmolytes were associated with abiotic stress tolerance in plants. As suggested by Molla[9], the higher accumulation of proline might be helpful to for better osmotic maintenance under water stress. Although proline is regarded as an osmoprotectant under several stress conditions, there is a recent concept that proline accumulation is linked with detoxification of ROS[10]. The plants produce a large variety of specialized metabolites that appear to have no direct function in their growth or development but can function as defense against biotic and abiotic stresses. Specialized metabolites, such as phenol and flavonoids are accumulated under drought stress in some species[11–13], suggesting that they may be envolved in the

ROS-scavenging system and drought resistance.

The specie *A. cruentus* is important as a nutritional food because both the leaves and seeds can be used. The leaves of the young *A. cruentus* plants may be used in salads and soups and the grains are utilized into production of breads, cakes, cookies and added to soups[14]. In addition to the important nutritionally primary metabolites, amaranth plants also contain some specialized metabolites compounds that also play an important role in the human diet besides performing some special functions in the plants[15]. Due to the to the high nutritional value of *A. cruentus*, the consumption of this plant has been recommended in order to contribute to different benefits such as antioxidant activity, increase pro-vitamin A and anticancerogenic compounds in a healthy diet due to its the specific specialized metabolite profile[16]. Although there are data on the effect of drought and nitrogen availability on plant performance when applied alone, less information is available on their interactive effects. Since plant responses depend on the interaction between different environmental stresses, the aim of this study was to analyse the influence of nitrogen availability on the responses of *A. cruentus* to water stress. We hypothesized that nitrogen supply would modify the effects of water stress on plant physiology. Our results will be helpful for understanding the response of *A. cruentus* plants to future changes in global climate. Additionally, we evaluated the ability of plants to recover from water stress.

1 Results and Discussion

Water and nitrogen are the two main limiting factors for crop growth and productivity. As predicted, the climate may change and, as a consequence, there will be changes in water availability. Plants, in general, respond positively to increment in nitrogen supply[17] and negatively to water stress[18]. The sensitivity of plants to water stress may be altered by factors such as nitrogen availability. Data on the interactive effect of nitrogen and water availability on performance of amaranth plants are limited. Although the species of *Amaranthus* are able to survive in poor soil, in this study high nitrogen supply increased the above ground dry matter about 168% when compared to low nitrogen (Table 1). Six days of water stress were not enough to reduce dry matter of the above ground and no interaction between nitrogen and water stress was observed (Table 1).

Table 1. Shoot dry matter (g plant^{-1}), photosynthetic pigment content (g m^{-2}), relative water content (RWC; %) and the two-way analysis of variance of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress and 24 h of rehydration. Values are mean±SE of 10, 6 and 5 plants for shoot dry matter, photosynthetic pigments and RWC, respectively. Significance levels are: *, P<0.05; **, P<0.01; ***, P<0.001; NS, not significant, - not determined. The means followed by the same small letters (for W at a given N level) and capital letters (for N at a given W supply) are not significant different at P = 0.05.

Variables	End of stress				After 24 h of rehydration			
	LN+W	LN-W	HN+W	HN-W	LN+W	LN-W	HN+W	NH-W
Shoot DM	4.56±0.21	4.46±0.13	12.22±0.36	11.86±0.34	-	-	-	-
Chl *a*	0.108±0.004	0.103±0.004	0.326±0.012	0.293±0.014	-	-	-	-
Chl *b*	0.038±0.001	0.037±0.001	0.106±0.004	0.094±0.004	-	-	-	-
Chl *a*/*b*	2.858±0.024	2.795±0.017	3.076±0.046	3.105±0.051	-	-	-	-
Carotenoids	0.043±0.001	0.0445±0.002	0.108±0.004	0.100±0.004	-	-	-	-
RWC	91.0±2.3aA	91.4±1.5aA	96.7±0.5aA	84.2±4.2bA	92.7±0.6	94.0±0.2	92.8±0.5	91.6±1.1
Two-way ANOVA								
Source of variation	N	W	N*x*W		N	W	N*x*W	
Shoot DM	***	NS	NS		-	-	-	
Chl *a*	***	NS	NS		-	-	-	
Chl *b*	***	NS	NS		-	-	-	
Chl *a*/*b*	***	NS	NS		-	-	-	
Carotenoids	***	NS	NS		-	-	-	
RWC	NS	*	*		NS	NS	NS	

Nitrogen is a component of chlorophyll molecules. Therefore, an increase in the concentrations of these pigments is expected when the plants are supplied with more nitrogen. Chlorophyll *a* and chlorophyll *b* concentration were increased about 200% and about 189%, respectively ((Table 1) when compared to low nitrogen. Also, the ratio *a*/*b* was increased about 8% under high nitrogen as a result of higher effect on Chlorophyll *a* (Table 1) which suggests a higher Photosystem II activity. Carotenoids are accessory pigments for photosynthesis and play an essential role in the photoprotection of the photosynthetic

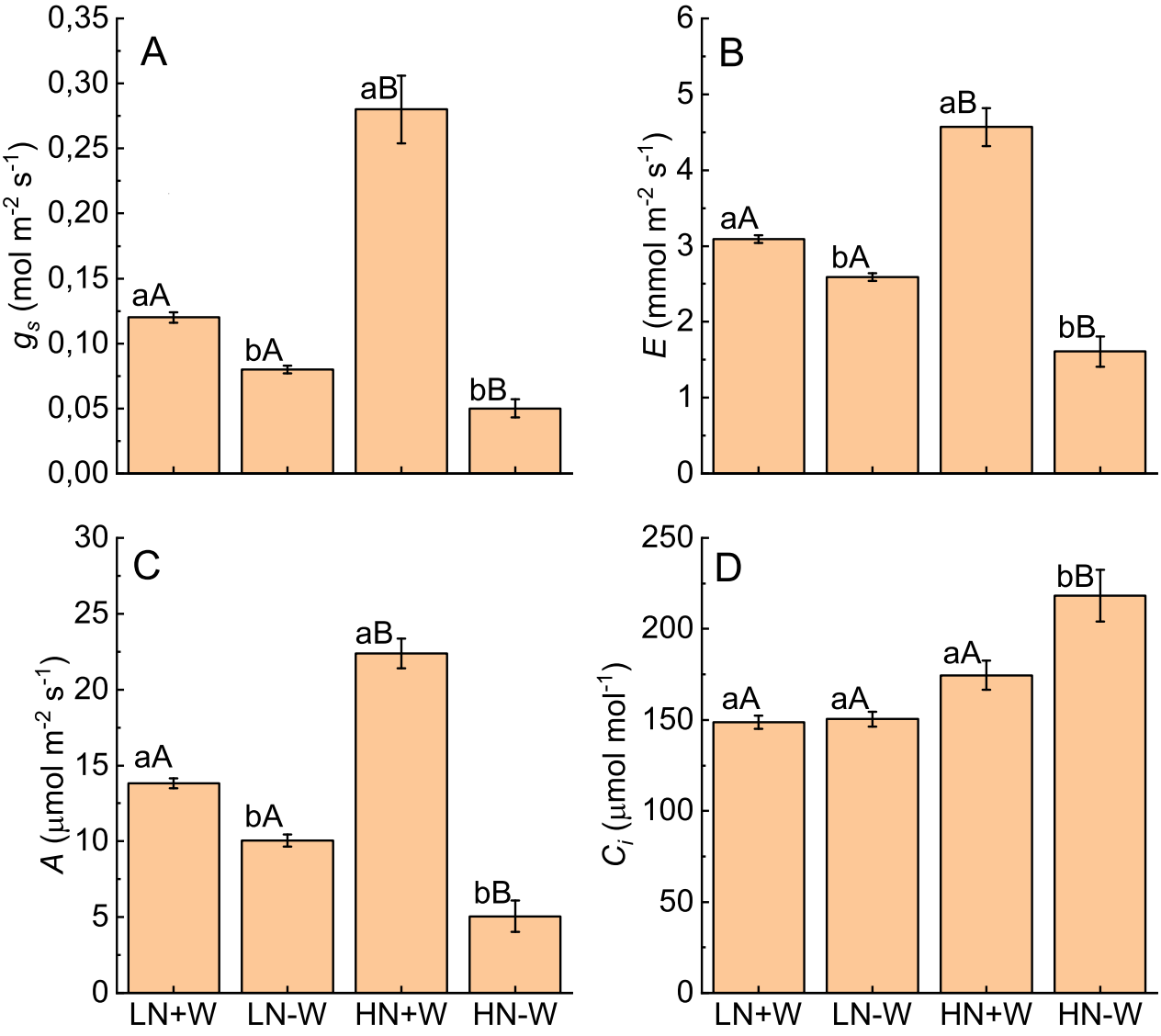

Figure 1. Stomatal conductance (g_s, A), transpiration (E, B), photosynthesis (A, C) and intercellular CO_2 concentration (C_i, D) of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress. Values are mean±SE of 9 plants. The means followed by the same small letters (for W at a given N level) and capital letters (for N at a given W supply) are not significant different at $P = 0.05$.

Variables	End of stress			24 h rehydration		
	N	Water	N*x*W	N	Water	N*x*W
g_s	**	***	***	***	NS	NS
E	NS	***	***	***	**	*
A	*	***	***	***	*	NS
C_i	***	*	*	NS	**	NS
WUE	NS	***	**	***	NS	*
Proline	***	*	*	***	NS	NS
Leaf N	***	NS	NS	***	NS	NS
Ammonium	***	NS	NS	***	NS	NS
Nitrate	***	**	**	***	NS	NS

Table 2. Two-way analysis of variance describing the influence of nitrogen (N) and water (W) supply on several characteristics of amaranth plants. Significance levels are as in Table 1.

apparatus. Higher nitrogen supply increased the concentration of carotenoids about 115% compared to low nitrogen. No effects of water stress and no interactions were found in these variables (Table 1). In some plants as maize subjected to drought stress there were reduction in chlorophyll *a* and chlorophyll *b* concentration[19]. The decline in chlorophyll content is accentuated as the period of stress increased from five to ten days[12]. As shown by Caeser *et al.*[20], the different levels of deydrations can induce different responses in terms of chlorophyll and carotenoids content.

As seen in Table 1, only water restriction affected the leaf relative water content (RWC) but due to the interaction between nitrogen and water stress, water stress reduced the RWC only in plants grown under high nitrogen after six days of water restriction. Lower nitrogen-grown plants after six days of drought maintained nearly the same RWC as the control. This difference in RWC between low and high nitrogen supply under water restriction arises as a consequence of increased leaf biomass under high nitrogen. The greater leaves biomass under high nitrogen represents greater area for transpiration and, as a consequence, faster consumption of water available in the substrate in addition to stimulation of the rate of transpiration and stomatal conductance (Figure 1A; B). After 24 hours of reydration, the RWC of high nitrogen-grown plants recovered to the same level as the non-stressed plants (Table 1).

The response of stomata to nitrogen supply has been widely studied, but the available results are contradictory. In the present study, high nitrogen increased the g_s about 133% of the plants grown under well water conditions (Figure 1A; Table 2) in comparison to low nitrogen. As observed by Matimati *et al.*[21], high nitrogen supply to roots enhances stomatal opening, provided that plants are well watered. An increase in g_s implies a greater availability of CO_2 in the sub-stomatal cavity but also a greater amount of water vapor lost through stomata. Restriction of water supply reduced g_s under both nitrogen supply but the interaction between the two factors resulted in a greater decrease in g_s under high nitrogen compared to low nitrogen. In general, changes in g_s are reflected in changes in the measured rate of transpiration (E). Although nitrogen as the main factor did not change significantly the E according to the analysis of variance, it interacted with water stress (Figure 1B; Table 2). Restriction of water supply reduced E under both low and high nitrogen supply, but due the interaction between nitrogen and water stress the reduction was higher under high nitrogen, similar to the results found by Song *et al.*[22]. The higher E per m^2 under high nitrogen and the higher leaf biomass resulted in more water lost per plant resulting in faster stress as seem in RWC. A small decline in g_s and E under mild water stress as observed under low nitrogen may have protective effects against drought by saving water since the RWC under this condition was not altered after six days of water restriction Table 1. The WUE was strongly reduced under water restriction and high nitrogen as a consequence of more negative effect on photosynthesis (A) than on E (Figure 3A; Table 2).

Higher nitrogen supply had a significant stimulated effect on A under well water supply, but the water stress drastically reduced it to values lower than in plants grown under low nitrogen independently of water supply (Figure 1C). These results are in agreement with those obtained by Song *et al.*[22] in *Populus* species. The higher drought effect under high nitrogen found in this study indicates that *A. cruentus* would be more sensitive to water restriction under this condition than under low nitrogen supply. Therefore, choose the appropriate nitrogen concentration may results in enhanced drought resistance of plants[23]. However, the response to nitrogen and water supply depend on plant species as found in *Phoebe zhennan* where nitrogen fertilization plays a crucial role in alleviating water stress damage[24]. The amount of CO_2 in the sub-stomatal cavity depends on the g_s and on the mesophyll conductance from sub-stomatal cavities into the chloroplasts and by metabolic processes. As seen

in Table 2, both nitrogen and water supply affected the intercellular CO_2 concentration (C_i), but due to the interaction effect water stress increased the C_i under high with no effect on plants under low nitrogen (Figure 1D). Restriction of water supply did not alter the C_i under low nitrogen. Conversely, water stress under high nitrogen induced an increase in C_i of 25% compared to non-stressed plants as a result of interaction between the two factors (Figure 1D; Table 2). Lower A under water stress are, in general, associated with lower g_s. Although the reduction in g_s could undoubtedly play a part in limiting CO_2 fixation it is not the primary cause of the decline in photosynthetic activity under high nitrogen. Indeed, C_i of high nitrogen stressed plants was increased suggesting that lower A is not a result of stomatal limitation. Although some authors have shown that water stress decreased the concentration of chlorophyll[22,25,26] which could be partially responsible for reducing A this is not the case in the present study. The effect of drought on amaranth A differs between nitrogen supply and may be partially interpreted with respect to the greater increase in leaves biomass, E and g_s. The grater of leaf biomass under high nitrogen implies in more area for transpiration and, consequently, representing faster depletion of available water stock in the substrate. These inferences are supported by Table 1, which demonstrates a significant reduction of RWC under high nitrogen supply when compared to low nitrogen. Also, the rise in C_i under high nitrogen and low water supply suggests that the photosynthesis became also under control of mesophyll metabolism, which reduced the demand for CO_2. The inhibition of A can also occur due to a reduction in mesophyll conductance pathway from the substomatal cavities to the sites of carboxylation resulting in lower chloroplastic CO_2 partial pressures[27] in addition to reduction in photochemical efficiency of PSII as found in maize, another C_4 plant[19]. Reduced A was also shown to be associated to reduction in activity of Rubisco and the activities of enzymes involved in the C_4 pathway[28,29]. After 24 h of reydration, the g_s fully recovered under both low and high nitrogen supply while E under high nitrogen was increased about 29% when compared to non-stressed plants (Figure 2A, B; Table 2). The stimulation of E under high nitrogen could be explained as an attempt to reestablish the RWC. The rate of A fully recovered in low nitrogen-grown plants after 24 h of reydration while in high nitrogen-grow plants the A showed a small increase when compared to its control (Figure 2C; Table 2). Although not significant, the C_i was reduced under low and high nitrogen supply after 24 h of reydration. The reduction mainly under high nitrogen can be explained by the stimulation of A thus rapidly consuming the C_i available (Figure 2D; Table 2). The WUE was not fully recovered under high nitrogen due to the significant increase in E compared to A (Figure 3B). The fact that A was fully recovered after reydration indicates that damage to the photosynthetic apparatus did not occur under this level of water stress.

In high nitrogen-grown plants under well water condition, the total leaf nitrogen content increased 114% (Figure 4A; Table 2)) while A increased about 62% compared with low nitrogen supply (Figure 1C). The stimulation of A can be attributed to the high investment of nitrogen into the photosynthetic machinery such as chloroplast development[30] and more nitrogen allocation into photosynthetic enzymes Rubisco and PEPcase as observed in amaranth plants by Tazoe et al.[31]. Xiong *et al.*[32] found that drought stress for seven days significantly decreased the leaf transpiration and also shoot nitrogen concentration but this finding is inconsistent with those observed in this study. Since the restriction of water did not reduced the photosynthetic pigments neither the leaf nitrogen content, the results suggest that a dysfunction in A is not a result of lower leaf nitrogen or the presence of less photosynthetic pigments, but due to biochemical and/or photochemical limitations in addition to any stomatal conductance. The mechanistic basis for the lower rates of photosynthesis under high nitrogen and water stress has yet to be elucidated. It is interesting that under well nitrogen nourish plants which present high levels of leaf nitrogen were not able to cope with the effect of water stress. It seems that under this condition the lower RWC in the leaves may compromise the activity of the enzymes involved in photosynthesis as pointed out by some authors[28,29]. After 24 h of rehydration, no change in leaf N content was observed when compared to stressed conditions (Figure 4B).

Proline works as both an osmoprotectant and as a redox-buffering agent possessing antioxidant property under conditions of stress[33]. Accumulation of proline under drought stress was found in several plants[26], particularly in young leaves[34]. The accumulation in proline becomes greater as the stress period is increased[12], suggesting that the change in osmotic potential is important to keep various physiological processes functioning. Besides that, proline was found to be the major non-enzymatic antioxidant metabolite under water stress conditions, resulting in stress tolerance as a consequence of its ROS-scavenging ability[35]. Since proline is an amino acid which contain nitrogen an increase in its synthesis was expected under high nitrogen supply (Figure 5A; Table 2). At lower nitrogen, the difference between well watered and stressed plants was smaller and not significant. However, this difference became grater as the nitrogen was increased. It is not surprising that at low nitrogen there was no significant increase in proline since the water stress in this condition was slowly with no significant changes in RWC. Therefore, it seems likely that the increase in proline is a protection mechanism against the harmful effects of drought. The beginning of the accumulation and the amount of proline accumulated under drought was found to be dependent on the *Amaranthus* species and genotypes sensitivity and the degree of water stress[25,36] and also dependent of nitrogen level as found in rice leaves[37]. After 24 h of rehydration, the level of proline was reestablished, which means that there was only the effect of nitrogen (Figure 5B; Table 2).

Nitrate and ammonium are the main nitrogen sources for plants with nitrate being the preference of most species[38]. Under optimum nitrogen availability, an increase in nitrate content was found in leaves cells of *A. cruentus*[39]. Vacuoles are the major

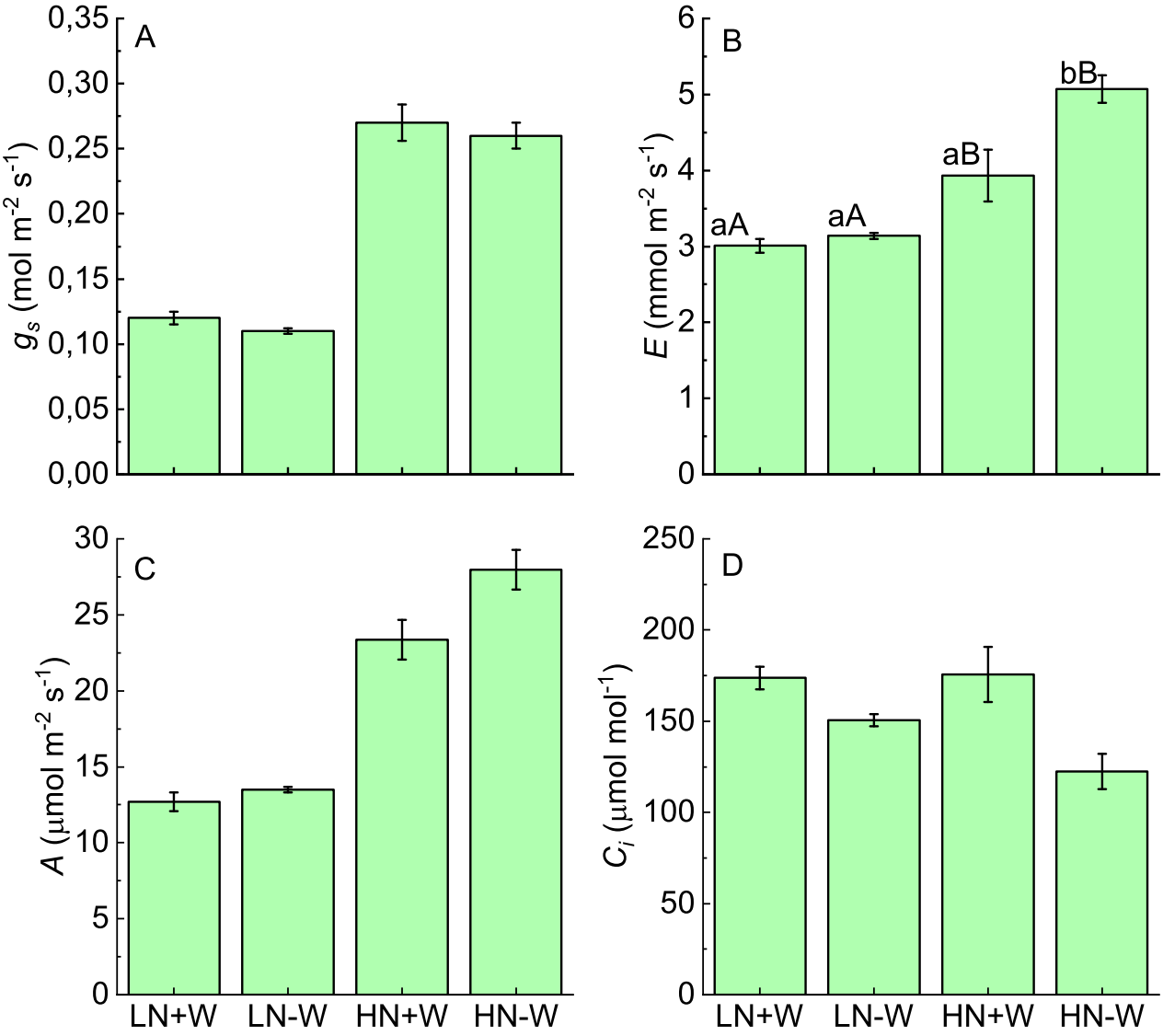

Figure 2. Stomatal conductance (g_s, A), transpiration (E, B), photosynthesis (A, C) and intercellular CO_2 concentration (C_i, D) of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 24 h of rehydration. Values are mean±SE of 9 plants. The means followed by the same small letters (for W at a given N level) and capital letters (for N at a given W supply) are not significant different at $P = 0.05$.

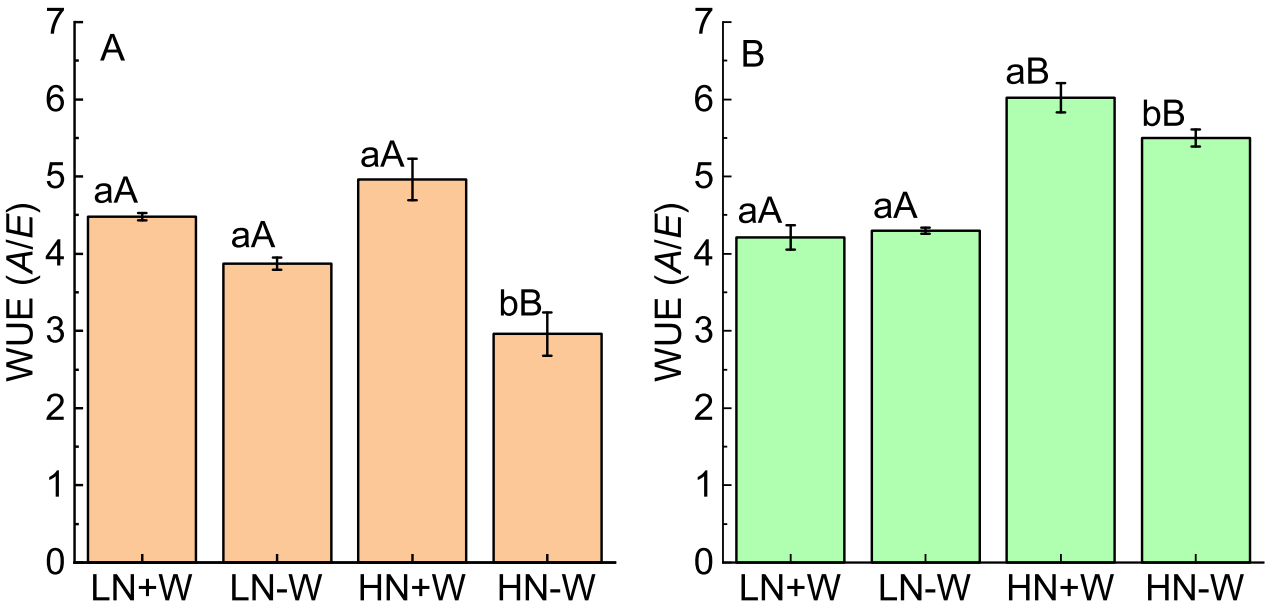

Figure 3. Instantaneous water use efficiency (WUE) of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress (A) and 24 h of rehydration (B). Values are mean±SE of 9 plants. The means followed by the same small letters (for W at a given N level) and capital letters (for N at a given W supply) are not significant different at $P = 0.05$.

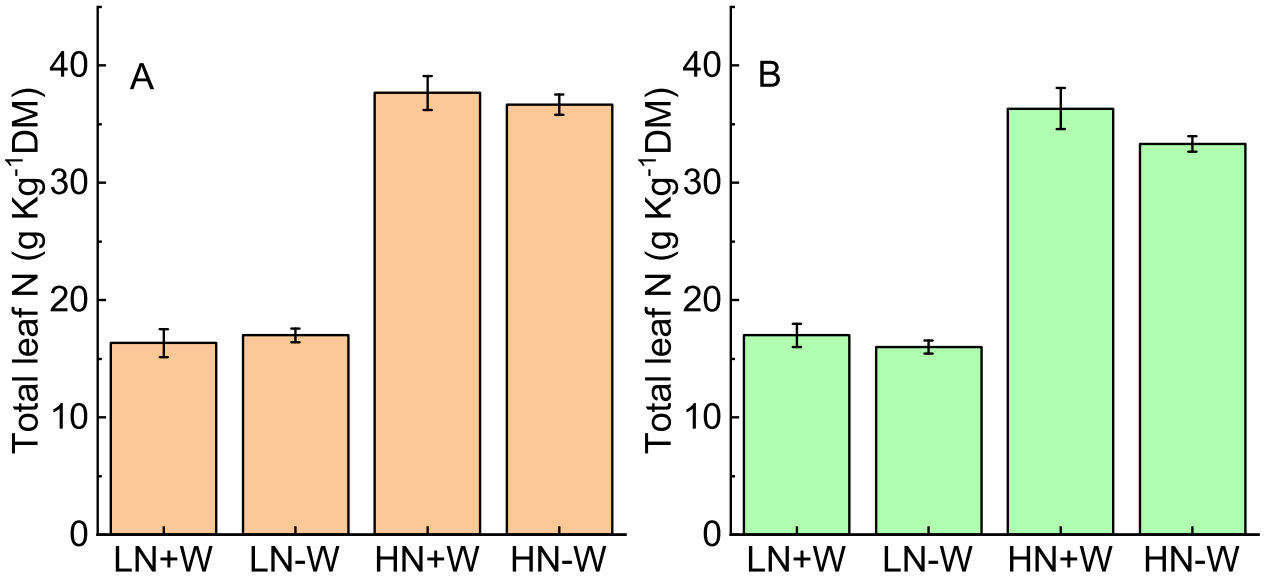

Figure 4. Total leaf nitrogen concentration of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress (A) and 24 h of rehydration (B). Values are mean±SE of 3 plants.

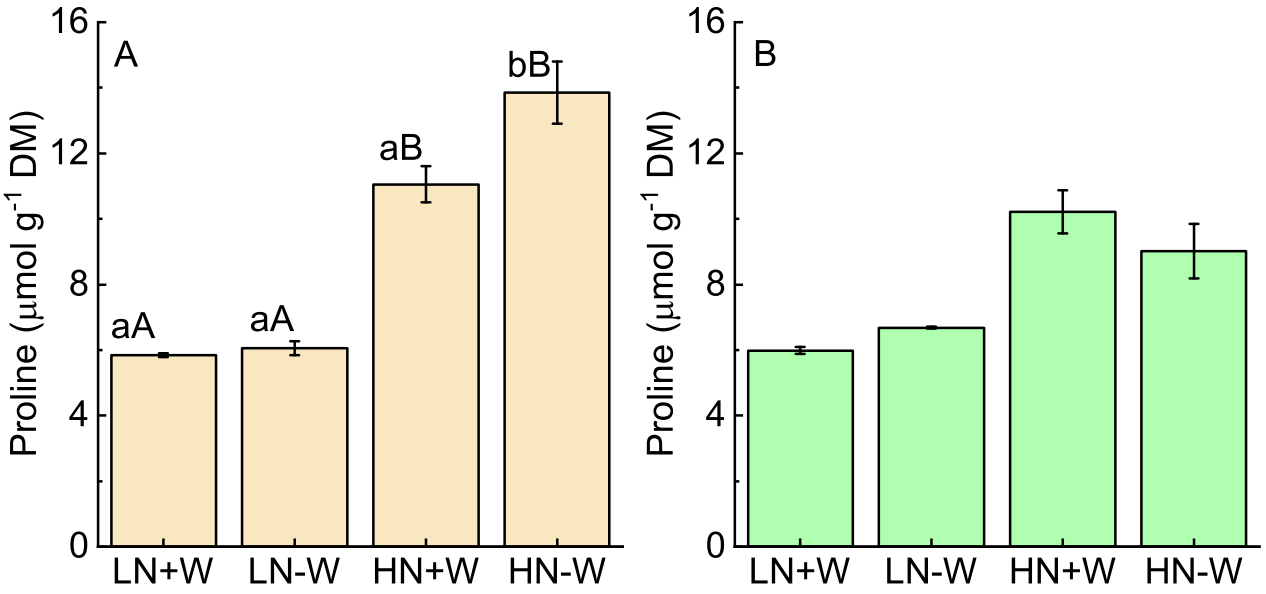

Figure 5. Proline concentration of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress (A) and 24 h of rehydration (B). Values are mean±SE of 4 plants. The means followed by the same small letters (for W at a given N level) and capital letters (for N at a given W supply) are not significant different at $P = 0.05$.

nitrate storage pool[40] and it can be used when nitrogen is available below the need for growth. In the present study, large accumulations of leaf nitrate were found when the plants were supplied with higher nitrogen due to an excess in the absorption as a consequence of greater availability of nitrogen in the soil in relation to the transformation capacity into organic compounds (Figure 6A; Table 2). The leaves of *A. cruentus* presented an accumulation of nitrate of 579.5 mg kg[1] of dry mass. After six days of water stress, high nitrogen-grown plants attained a lower concentration of nitrate than the corresponding leaves of not stressed plants with no effect on low nitrogen (Figure 6A; Table 2). Although the plants received equal doses of nitrate and ammonium, the accumulation of ammonium in the leaves was lower than nitrate (Figure 6C; Table 2). The increase in ammonium indicates saturation of the capacity of the enzymes involved in the assimilation of nitrogen into organic compounds. Although high levels of ammonium are considered toxic to plants resulting in growth reduction[41], high levels of ammonium in the leaves of *A. cruentus* did not result in visible toxic effects after 6 days of water restriction.

Several environmental factors such as drought can alter nitrogen uptake and assimilation in plants because water is required for nitrogen absorption and utilisation, suggesting that the plants can become simultaneously water and nitrogen limited[42]. Indeed, as observed by Wang *et al.*[43], higher stomata apertures in drought-resistant cultivar of apple plants enhanced transpiration rate, which promoted more nitrogen uptake. Relative higher xylem secretion rate and transpiration rate, which is the major pathway of water loss, were observed at moderate and high nitrogen supply but under water stress they were significantly suppressed at all nitrogen levels[37], with no alteration in total leaf nitrogen. As found by Zhong *et al.*[37], the greater reduction in *E* under high nitrogen and water stress did not resulted in alteration of total leaf nitrogen in amaranth but altered the concentrations of proline and nitrate. This may indicate that under high nitrogen and water stress there is a change in allocation of nitrogen to proline and to others nitrogen compounds. Water stress and nitrogen metabolism have interactive effects and, consequently, nitrogen metabolism affects a series of physiological and biochemical changes of great significance for plant tolerance to drought[44]. The enzyme activity of nitrogen metabolism was higher under an adequate nitrogen supply when compared to low availability but the activity of nitrate reductase (NR) was reduced by water stress under both nitrogen supply[19]. Song *et al.*[22] observed that the nitrate content and NR activity of maize leaves were significantly reduced under drought stress, while moderate nitrogen supply promoted the accumulation of nitrate and an increase in the NR activity. They concluded that moderate nitrogen supply increases plant resistance to drought stress, while high or low nitrogen concentrations increase the sensitivity of maize to drought stress.

Xiong *et al.*[32] found that there was a significant interactive effect between nitrogen and water stress on nitrate and ammonium content in the leaves with high nitrogen-grown plants presenting significantly lower and higher content under drought stress, respectively. The authors attributed to the accumulation in ammonium in the leaves under drought and high nitrogen to an increment in NR activity and a reduction GS activity, revealing that an increased accumulation of ammonium under drought might be attributed to the enhanced nitrate reduction. This is not the case in this study since under high nitrogen

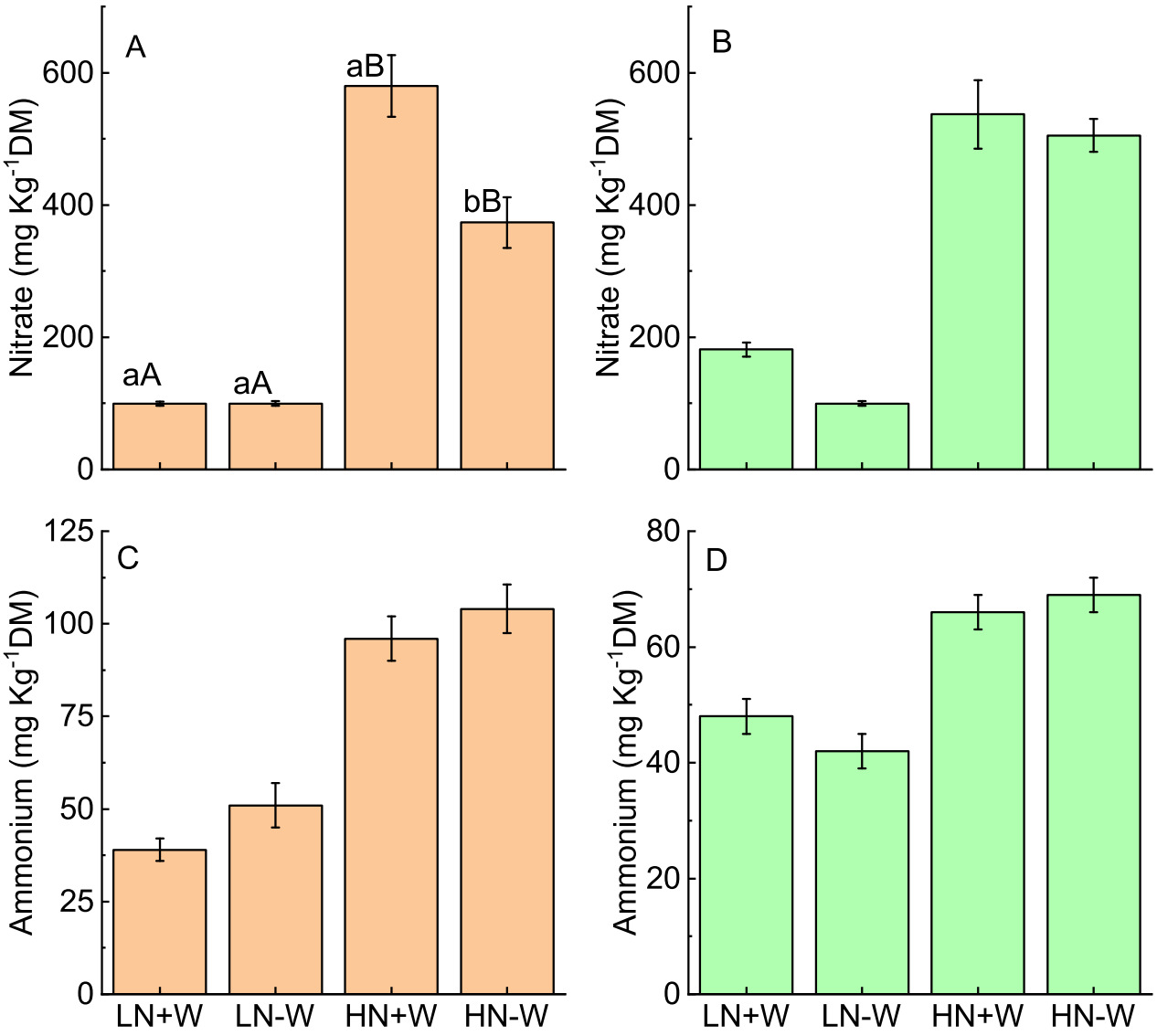

Figure 6. Leaf nitrate (A, B) and ammonium (B, C) concentrations of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress (A, C) and 24 h of rehydration (B, D). Values are mean±SE of 3 plants. The means followed by the same small letters (for W at a given N level) and capital letters (for N at a given W supply) are not significant different at $P = 0.05$.

and water restriction the lower nitrate content was not accompanied with an increase in ammonium in the leaves. It seems that, due to the fact that there was no change in the content of total leaf N, there was a deviation of N to the proline synthesis. Slabbert[36] suggested that proline may be act as a storage compound for carbon and nitrogen during water stress when the synthesis of starch and protein are inhibited. Additional research is needed in order to clarify this response in *A. cruentus* plants. There is abundant evidence that water deficit can alter the nitrogen metabolism but the effect vary accordingly to the species and the intensity of stress. For example, in *Hippophae rhamnoides*, the two levels of drought stresses (50% and 30% field capacity for 12 weeks) decreased the absorption of ammonia and nitrate nitrogen, whereas only inhibited nitrate absorption of *H. thibetana*[45]. Also as found by Huang *et al.*[46], the net influx of nitrate at the root surface was lower in response to drought stress while the influx of ammonium tended to increase along the fine roots of PEG-treated *Malus prunifolia*. These findings explain the lower nitrate in the leaves of *A. cruentus* but not the level of ammonium which remained unchanged under drought stress. After 24 h of rehydration, the level of nitrate under high nitrogen was reestablished (Figure 6B; Table 2). In the present study, neither water stress nor the reydration altered the level of ammonium in the leaves (Figure 6C, D; Table 2).

The available resources such as nitrogen, carbon and sulphur will have impact on the production of specific classes of primary metabolites which in turn synthesized specialized metabolites indicating super-coordinated gene expression networks connecting primary and specialized metabolism in plants[47]. The photosynthetic capacity is negatively correlated with the leaf phenolic concentration[48] and this has been suggested to represent the gradient between a maximum carbon gain and maximum protection[49], reflecting the trade-off between growth and defence/protection demands, depending on the growth strategy adopted by each species[48]. The phenolic compounds which are found mainly in the vacuole of several cells types in the leaves act as multifunctional specialized metabolites under abiotics stress[49], such as non-enzymatic antioxidants[50] and repair of membrane from lipid peroxidation[51]. Several author have found that water stressed plants showed an increase in total phenols and flavonoids[20,26], which suggests that the increased synthesis of these substances by plants represents an important defense mechanism of drought tolerance[11]. As it has been pointed out, the biosynthesis of flavonoids occurs predominantly when the antioxidant enzymes are inactivated[52].

In the present study, the low and high nitrogen grown-plants of *A. cruentus* were stressed by stopping the irrigation completely for six days. The representative chromatograms for each experimental group are shown in the Supplementary Material. Peaks in the chromatogram were identified as phenolic acids and as flavonoids based on comparison of absorption spectra in UV (Supplementary Material) with the available standards. The areas of each of the detected peaks were obtained and the calculations extrapolated, and the quantification expressed in μg mg^{-1} of dry extract. The average obtained for each experimental group and classes of substances are presented in (Table 3). The coefficient of variation which is a measure of dispersion of the variables were, in general, high (Table 3) which means that the higher the coefficient of variation, the greater the level of dispersion around the mean. The two-way analysis of variance presented in the Table 3 show that the water stress did not change significantly the content of phenolic acids, total phenols or flavonoids. The reason for this response might be due to the duration and intensity of the stress as pointed out by Mahajan *et al.*[53]. On the contrary, high nitrogen reduced the concentration of flavonoids and total phenols independently of water stress. Total phenols concentration of *Beta vulgaris* plants under N-starvation conditions was higher in the leaves and roots, in comparison to standard nitrogen supply[54]. This result is similar to those found in this study for amaranth which belongs to the same family. The effect of increasing nitrogen on *Lactuca sativa* plants not only reduces the content of phenolic compounds but decreased every type of phenolic compounds[55]. Since photosynthetic capacity is negatively correlated with the leaf phenolic concentration[48] this can partially explain the lower concentration of total phenols and flavonoids under high nitrogen supply in this study. After 24 h of reydration, only nitrogen had a small significant effect on the content of phenolic acids and flavonoids (Table 3). The content of phenolic acids presented a small increase while flavonoids remained lower after 24 h of rehydration when compared with low nitrogen supply.

In summary, the regulatory function of nitrogen in drought tolerance of plant depends upon the intensity of stress, nitrogen level, and plant species. High nitrogen supply resulted in better performance of *A. cruentus* plants. Indeed, under this satisfactory condition plants did not need to invest in secondary defense metabolites such as phenolic compounds which were reduced under this condition. However, a short time of water restriction (six days) caused a significant reduction in RWC, gas exchange characteristics, WUE and leaf nitrate but the proline concentration was increased, and it was not able to increase phenolic compounds. The observed interaction between nitrogen and water supply resulted in no alleviation of the effects of water stress on *A. cruentus*, on the contrary, it increased it. Taken all together the study presented here suggests that the responses of amaranth to water stress are likely to be influenced by nitrogen status. Given the importance of amaranth plant as a food and the need to use nitrogen fertiliser to increase crop productivity, a fuller understanding of the interaction effects between nitrogen and water supply is desirable. Additional studies, especially regarding the possible physiological and biochemical changes in amaranth plants due to the interaction between nitrogen and water supply, are necessary.

Variables	End of stress				After 24 h of rehydration			
	LN+W	LN-W	HN+W	HN-W	LN+W	LN-W	HN+W	NH-W
Fenolic acids	26.91±1.46	42.17±2.76	33.41±1.94	29.73±9.09	21.28±5.51	24.62±2.75	31.19±3.51	32.42±0.77
Flavonoids	53.01±1.95	44.96±2.92	32.91±1.13	26.02±6.94	36.08±8.90	38.27±2.26	24.99±2.48	20.99±7.47
Total phenols	80.01±0.55	87.13±4.59	66.32±0.81	55.75±14.56	57.37±8.55	62.89±5.01	56.18±5.75	53.41±7.84
Coefficient of variation (%)								
Fenolic acids	9.4	11.3	10.0	52.9	44.8	19.7	19.5	4.1
Flavonoids	6.4	11.2	5.9	46.0	42.7	10.2	17.2	61.2
Total phenols	1.2	9.0	2.1	45.0	25.8	13.8	17.0	25.4
Two-way ANOVA								
Source of variation	N	W	N*x*W		N	W	N*x*W	
Phenolic acids	NS	NS	NS		*	NS	NS	
Flavonoids	**	NS	NS		*	NS	NS	
Total phenols	*	NS	NS		NS	NS	NS	

Table 3. Content of phenolic acids, flavonoids and total phenols in μg/mg of 85% MeOH leaf extract, the coefficient of variation and analysis of variance of amaranth plants grown under low nitrogen and good water supply (LN+W), low nitrogen and low water supply (LN-W), high nitrogen and good water supply (HN+W) and high nitrogen and low water supply (HN-W) after 6 days of water stress (A) and 24 h of rehydration. Significance levels are as in Table 1.

2 Material and methods

2.1 Plant material and growth conditions

BRS Alegria is a new cultivar of *A. cruentus* developed by the Center for Agricultural Research of Cerrados which was originated from the *A. cruentus* strain AM 5189 from the USA[56]. The seeds were purchased from the Brazilian Agricultural Research Company (Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA) which is a Brazilian government company and is the owner of the Center for Agricultural Research of Cerrados. The seeds of *A. cruentus* were sown in 4 L plastic pots filled with vermiculite and kept in a greenhouse under natural photoperiodic conditions and minimum and maximum average temperature of 16 and 33 °C, respectively. The plants were watered with 70% of full-strength nitrogen-free Long Ashton solution[57], containing different doses of nitrogen as ammonium nitrate. The nitrogen doses used were: 1.97 and 14.82 kg N ha^{-1}, which correspond to 20 and 100% of full-strength Long Ashton nutrient solution, respectively. The plants were supplied with 300 mL of nutrient solution per pot three times a week, and with water on the other days. The plants were stressed by suspension of watering after after 42 days of growth. After 6 days of stress imposition, half of the stressed plants of both low and high nitrogen supply were rehydrated for 24 h, in order to determine their ability to recover from the water stress.

2.2 Gas exchange measurements

A portable infrared gas analyzer (LCpro, ADC, Hoddesdon, UK) was used for measurements of photosynthesis (A), stomatal conductance (g_s), transpiration (E) and intercellular CO_2 concentration (C_i) on the youngest fully expanded leaf after 6 days of water stress imposition and after 24 h of rehydration. Measurements were taken between 8 and 10 am inside the greenhouse under ambient temperature, partial pressure of carbon dioxide and water vapor pressure of air. Photosynthetic active radiation (PAR) of 1000 μmol m^{-2} s^{-1} was supplied by a light unit, mounted on top of the leaf chamber. The leaf was kept under this PAR until a steady-state rate was achieved. Instantaneous water use efficiency (WUE) was obtained as the ratio of photosynthetic carbon assimilation to water lost by transpiration (A/E).

2.3 Relative water content determination

The leaf relative water content (RWC) was determined in four leaf disks of known area per plant after six days of water stress and after 24 h of rehydration according to the following equation:

$$\text{RWC}\ (\%) = \frac{\text{(FW-DW)}}{\text{(TW-DW)}} \times 100\,, \tag{1}$$

where FW is the fresh weight obtained immediately after the removal of leaf discs; TW is the turgid weight determined after rehydration of the discs for 3 h and DW is the dry weight obtained after drying the discs in an oven at 60°C for 48 hours.

2.4 Photosynthetic pigments and leaf nutrients analysis

The photosynthetic pigments were measured on three leaf discs of known area from the same leaf used for gas exchange measurements. Pigments were extracted in 80% aqueous acetone and the content was calculated according to the equations

proposed by Lichtenthaler[58]. The proline content was determined according to the method described by Bates *et al.*[59] and modified by Torello and Rice[60] from oven dried and finely powder leaves and the concentration expressed on a leaf dry weight basis by using proline as standard. The determination of nitrate, ammonium and total nitrogen was made in the leaves located in the middle region of the plant. The leaves were oven-dried at 60°C until constant weight was obtained. The leaves were then finely ground with a mill and sent for analyses at Soil Laboratory of School of Agricultural Sciences, Botucatu, São Paulo/Brazil. The foliar nitrogen, nitrate and ammonium analysis were performed by the semi-micro-Kjeldahl method, after sulphurous digestion of dried and finely ground leaves according to Malavolta *et al.*[61].

2.5 Leaf phenolic content analysis

2.5.1 Extract preparation

Leaf samples from 24 specimens of *A. cruentus* grown in different cultivation conditions were collected in triplicate and hot air dried 60°C. The dried leaves were powdered using a knife mill and an aliquot of 50 mg was extracted using MeOH:H_2O 85:15 (v/v), via ultrasound for 30 minutes at room temperature. The supernatant was collected and centrifuged at 3000 rpm. The obtained solution was subjected to solid phase extraction in reverse phase (SPE-C_{18}) cartridges followed by filtration in PTFE membrane (0.45 μm). The samples were analyzed randomly at a final concentration of 3.5 mg ml^{-1}.

2.5.2 HPLC-PAD analysis

The extracts were analyzed in a high performance liquid chromatograph (PU-2089S Plus, Jasco®) coupled to a photodiode array detector (MD-2015 Plus Jasco®) and automatic injector (MD AS-2055 Jasco®). Chromatographic separations were performed on a Phenomenex® Luna C_{18} column (250 x 4.6 mm d.i., 5 μm) at 40°C. The mobile phase system consisted of a MeOH and ultrapure water, acidified with 0.1% Formic Acid (Synth®). The samples were analyzed using a linear gradient as follows: 5 to 65% of MeOH in ultrapure water in 65 minutes. The flow rate was 1 ml min^{-1}.

2.5.3 Quantitative determination of constituents

The determination of the content of phenolic compounds was performed using the method of external standard. Quantification of the constituents was performed using a regression curve each standard injected in triplicate. Measurements were performed at 280 for phenolic acids and 360 nm for flavonoids. The total phenol contents were obtained from the sum of the quantified values for phenolic acids and flavonoids, in their respective extracts. The Jasco ChromPass software (Version 1.8.1.6) was used to process the chromatograms.

2.6 Dry matter determination

At the end of water stress treatment imposition (49 days after sowing), the plants of each treatment were selected randomly for shoot dry matter determination. The amaranth plants were divided into stem and leaves before being oven dried at 60°C for at least 48 h.

2.7 Statistical Analysis

The data were subjected to a Factorial Analysis of Variance (2x2) using SPSS/PC for Windows 9.0. Nitrogen levels and water levels were treated as fixed effects and a univariate test was performed on each variable separately at the 5% level. Least Significant difference procedure (LSD) for comparing treatment means was only performed for the variable with significant F-test for the main effects (N and water) and/or their interaction.

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Author contributions statement

I.C. conceived and supervised the problem. All authors contributed to the experimental work and analysed the data. I.C. wrote the manuscript. All authors read, corrected and approved the manuscript in its final form.

Additional information

The authors declare no competing interest. The authors also declare that the plant experiments were performed in accordance with national/institutional guidelines and regulations.

Availability

The data produced in this experiment can be obtained freely by request to the corresponding author.

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